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Published in:
Journal of Agricultural Science

DOI:
10.1017/S0021859616000423

First published: 28/07/2016

Variation in grain skinning among spring barley varieties induced by a controlled environment misting screen

Short title: G*rain skinning among spring barley varieties*

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(MS received 11 November 2015, revised 12 April 2016, accepted TBC May 2016)

SUMMARY

The current study investigated use of a controlled misting environment to simulate field conditions that have been implicated in high levels of the malting barley defect, grain skinning. More than 200 spring barley varieties were assessed to identify those varieties that were particularly resistant or susceptible to the defect. Relationships between skinning severity and the traits ear length, floret number, grain number and grain weight were examined among the varieties. In a panel of seven varieties chosen as treatment controls, misting was found to significantly increase skinning severity. The misting treatment had no effect on measured ear traits of these varieties. Among the 200 varieties grown under the misting treatment, there was a continuous spectrum of skinning severities, which were not correlated with ear length, floret number, grain number or grain weight. Using the misting treatment, differences in susceptibility to grain skinning could be determined among varieties. As the misting treatment did not affect measured ear traits, and no correlation was found between ear traits and skinning severity among varieties, the effect of misting on skinning severity must be mediated through other physiological characteristics.

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INTRODUCTION

Spring barley (*Hordeum vulgare*) for malting purposes must meet set quality requirements to maximize malting efficiency. If a batch of barley fails to meet these requirements it may be rejected at a maltings. The barley grain comprises an outer husk and an underlying caryopsis, to which the husk is firmly attached at harvest. When the husk is partially or wholly detached from the caryopsis, the grain has skinned. Grain skinning, sometimes referred to as 'hull peeling', is a quality defect in malting barley. Good quality adhesion of the husk to the caryopsis confers several advantages to malting efficiencies because germination of the barley grain is a key step in malting. The husk prevents germination losses by protecting the embryo from mechanical damage during harvest and post-harvest handling. Grains with intact husks also maintain better germination vigour during storage, over grains that have skinned (Mitchell *et al.* 1958). Skinned grains not only have lower germination rates, but reduced saccharifying activity, leading to malting losses (Meredith 1959). Skinned grains that do germinate imbibe water more quickly than those with firmly adhering husks; such grains germinate earlier than grains with intact husks and therefore over-modify in a batch of malt, reducing potential malt extract (Bryce *et al.* 2010).

Grain skinning can be assessed in different ways, but is typically based on the segregation of grains that have lost an area of husk above a chosen threshold, followed by either counting or weighing the proportion of these grains. Assessing grain skinning is subjective as there is currently no means of quantitatively measuring skinning, although good consensus can be achieved using a threshold approach (Olkku *et al.* 2005). Studies using such thresholds have shown that skinning is a heritable trait, but one that is largely influenced by environment (Aidun *et al.* 1990). Although differences in skinning levels have been observed among genotypes, typically only a small number of genotypes have been compared within any one study. Skinning assessments on harvested grains indicates that environmental conditions and year of harvest have higher influence on skinning than genotype, whereas for malted grains, genotype explains a higher proportion of the variation in skinning than harvest location or year (Legge *et al.* 2005; Psota *et al.* 2011). The severity of skinning is exaggerated by physical handling (Olkku *et al.* 2005; Reinbergs & Huntley 1957); therefore the proportion of skinned grains increases throughout the malting process (Legge *et al.* 2005; Olkku *et al.* 2005), which is likely to make genotypic differences more easily quantified.

As the fundamental causes of skinning are not currently known it is difficult to select against the condition in barley breeding programmes. Grain size was hypothesised to have an effect on a cultivar's tendency to skin by challenging the mechanical strength of the outer grain tissues (Rajasekaran *et al.* 2004), yet no correlations between grain skinning and grain plumpness or weight have been found to date in studies comparing up to 16 varieties (Legge *et al.* 2005; Rajasekaran *et al.* 2004). Production of a lipid cementing layer is required for adhesion of the husk to the barley caryopsis (Gaines *et al.* 1985; Harlan 1920), and is controlled by the NUD (NUDUM) transcription factor which regulates expression of genes involved in lipid biosynthesis (Duan *et al.* 2015; Taketa *et al.* 2008). Naked barleys do not produce a cementing layer; this is a different phenotype compared with skinning, in which the lipid cementing layer is produced but there is a failure in the quality of husk adhesion. Skinning may be mediated through changes in the structure (Hoad *et al.* 2016) or composition of this lipid layer and knowledge of plant cuticle structure provides some clues as to how such changes could be facilitated. Plant cuticle structure and composition are influenced by genotype, but also by environmental factors such as radiation, temperature and moisture (Shepherd & Griffiths 2006). Indeed, growing seasons with cyclical rainfall causing repeated wetting and drying during grain filling have been associated with high levels of grain skinning. Field trials replicating such a season by 'sprinkling' plants with water during the growing season found that sprinkled plants had significantly increased skinning levels over non-sprinkled plants (Froment & South 2003); however there is still a need for a reproducible means of inducing grain skinning in order to determine differences in susceptibility to the condition among genotypes.

Due to the challenges involved in accurate quantification of grain skinning, and the high variability of the condition due to genotype-environment interactions, it can be difficult to assess genotypic susceptibility or correlations with other grain traits by comparing small numbers of varieties; the conclusions drawn from such studies may be biased depending on the varieties chosen. The current study aimed to investigate whether a controlled programme of misting during grain filling could be used to induce high levels of grain skinning. The misting treatment was then used to identify differences in susceptibility to grain skinning among more than 200 elite spring barley varieties. Correlations between grain skinning and the varietal characteristics ear length, floret number, grain number and grain weight were investigated to determine whether varietal differences in grain skinning susceptibility were associated with variation in these traits.

MATERIALS AND METHODS

Plant growth

A selection of 216 two-row spring barley varieties were chosen for skinning assessment from the Association Genetics of UK Elite Barley (AGOUEB) germplasm collection (http://www.agoueb.org/) maintained by the James Hutton Institute (UK). One hundred of the above varieties were grown in a glasshouse compartment in 2013 (Gh13), with the remaining 116 varieties grown under the same conditions in 2014 (Gh14). Varieties in Gh13 included many with high commercial relevance in the UK and Europe, whereas those in the Gh14 group represented a more diverse range of elite varieties. Seeds were sown in spring directly in Levingtons No. 2 compost, at a density of seven plants of each variety in 4-litre pots. Varieties were grown in duplicate pots on opposing sides of a glasshouse compartment maintained at a minimum temperature of 10°C, with a mean temperature of 18°C throughout the growing period. From anthesis to ripening plants were subjected to a fine mist of water from overhead sprayers controlled by an electronic timer for 1 min three times a day (09.00 h, 13.00 h and 17.00 h), delivering 3 mm water per day. Included in each of the Gh13 and Gh14 varieties was a selection of seven varieties, commercially relevant to the malting industry, that were grown in both years under both the misting treatment and also without misting as treatment controls. The non-misted control pots were located in the centre of the same glasshouse compartment. These seven varieties comprised the following: Concerto, Glassel, Optic, Oxbridge, Prisma, Shuffle and Tankard. Natural daylight was supplemented with mercury vapour lamps so that the minimum photoperiod was 16 h with photosynthetically active radiation (PAR) at plant ear level at 150 µmol/m^2/s. Ten ears were harvested from each variety of the glasshouse-grown plants and measured as below, followed by threshing in a Wintersteiger LD 180 laboratory thresher (Wintersteiger AG, Ried, Austria) for 5 s/ear before assessing for grain skinning. Ears were harvested after reaching growth stage 92 (Zadoks *et al.* 1974) and stored in the laboratory post-harvest, reaching an approximate moisture content of 12% at the time of grain measurements and threshing.

Ear and grain measurements

Measurements were made on ears harvested from Gh13 and Gh14 before grains were scored for skinning. Ear length (mm) was measured from the peduncle to the tip of the topmost palea using a ruler and the total number of florets and filled grains were counted. The total mass of grains from each ear was weighed using a Mettler Toledo (Columbus, Ohio, USA) XP6 microbalance (accuracy ± 1 μg) before threshing.

Skinning assessment

Assessment of grain skinning was done according to an in-house protocol, developed with the Institute of Brewing and Distilling (Scottish Micromalting Group, The Maltsters' Association of Great Britain, Nottinghamshire, UK). All of the grains from each ear were examined individually and a threshold of one fifth or greater husk loss by area was used to determine whether a grain was skinned. Grains with less than one fifth husk loss by area were counted as intact.

Statistical analyses

Statistical analysis was undertaken using GENSTAT (GenStat 16th Edition. Release 16.1, VSN International Ltd., Oxford). A generalized linear mixed effects model (GLMM) was used to assess the effect of the misting treatment on grain skinning, with the logit link function used to relate the proportion of skinned grains (response variable) to the predictor variables. In the first instance, to determine whether the misting treatment could be used as an efficient screen to induce grain skinning, models were built using data only from the seven varieties grown under misting and control conditions in both Gh13 and Gh14. Firstly, to assess whether the left-hand side (LHS) was significantly different from the right-hand side (RHS) within the misting treatment, the fixed effects were glasshouse side (LHS or RHS) and variety, with the random effect being variety nested within block nested with the year. The LHS and RHS were not found to be significantly different. To determine whether skinning levels differed significantly between the two years, the fixed effects were year, variety and the interaction between year and variety with the random effect being variety nested within glasshouse location (LHS, RHS or centre) nested within year. Neither year, nor the interaction between year and variety had a significant effect on skinning levels. Therefore, to assess whether the misting treatment significantly affected grain skinning relative to the un-misted control treatment, LHS and RHS were treated as a block, with the fixed effects being treatment and variety, and the random effect was variety nested within the block factor of glasshouse side by year.

To determine differences in grain skinning among the 216 varieties, Gh14 and Gh13 were assessed separately. A GLMM was employed using the logit link function for skinning as the response variable as above, with LHS and RHS treated as blocks, variety as the fixed effect and variety nested within the block as the random effect.

The effect of the misting treatment on the grain and ear traits was assessed for each trait separately using the restricted maximum likelihood (REML) algorithm, with variety, treatment and their interaction as the fixed effects, and variety nested within the block factor of variety by year as the random effect.

Finally, a GLMM was employed on the combined Gh13 and Gh14 data to assess whether grain skinning (using the logit link function, as above) was directly related to the measured ear and grain traits. The fixed effects in each case were variety and the measured trait, and the random effect was glasshouse side by year as the block factor.

RESULTS

Effect of misting on grain skinning

Due to practical considerations, plants grown under misting conditions were located on the left and right side of a glasshouse compartment. To determine whether the misting treatment had an effect on grain skinning severity, seven of the 216 varieties with high commercial relevance were grown under non-misted (control) conditions in the centre of the same glasshouse compartment in both 2013 and 2014. These control varieties were Concerto, Glassel, Optic, Oxbridge, Prisma, Shuffle and Tankard. Firstly, differences between these seven varieties on the left- and right-hand side of the glasshouse misting treatments were tested to determine whether the treatment on both sides of the glasshouse was comparable, or whether the treatment gave significantly different levels of skinning on each side (location). Comparing the left- and right-hand side of the glasshouse for these seven varieties showed that variety had only a weak effect on skinning severity ($P = 0.07$), whereas location and the interaction between variety and location had no significant effect. Both the left- and right-hand side misting treatments were therefore considered comparable. The effect of the misting treatment on grain skinning compared to the non-misted plants was then tested among these seven varieties. The misting treatment was found to significantly increase skinning severity compared to the non-misted plants ($P \leq 0.01$), but differences among these seven varieties, and the interaction between variety and treatment, were not significant. Ears from plants grown under the control treatment had a mean proportion of skinned grains of 0.030, whereas ears from plants grown under the misting treatment had a mean proportion of skinned grains of 0.215.

Effect of misting on ear and grain measurements

Each of the measured ear and grain traits were found to differ significantly among the control varieties, but neither treatment, nor the interaction of treatment and variety, had a significant effect (model results and significant differences among varieties are given in Table 1). Generally, Glassel, Prisma and Tankard had shorter ear lengths with low grain number and grain weight compared with the varieties Concerto and Shuffle, which had longer ears with higher grain number and grain weight. Optic and Oxbridge had intermediate ear lengths and grain weights. Since the misting treatment had no effect on ear and grain traits, the increase in skinning severity caused by the misting treatment must be due to other factors.

Variation in skinning severity among varieties

The Gh13 varieties grown under the misting treatment in 2013 included many listed for malting and feed purposes in the Agriculture and Horticulture Development Board (AHDB) Recommended Lists for cereals in the UK. The mean estimated proportions of skinned grains for the 100 Gh13 varieties are shown in Table 2. A spectrum of skinning severity exists, with only varieties at either ends of the spectrum being significantly different from each other ($P < 0.05$). The variety Cork was an exception as it did not have any skinned grains, making it significantly different to all other Gh13 varieties examined ($P < 0.05$). Among the remainder of the varieties, those with low or high skinning severity (Table 2) were significantly different from each other ($P < 0.05$). However, the majority of varieties had moderate skinning severity and there were few significant differences between these varieties and those in the low or high severity categories. The Recommended lists from AHDB are available online from 2004 onwards (http://cereals.ahdb.org.uk/varieties.aspx). Since this date, Appaloosa was the only variety recommended for malting purposes that had low skinning. The majority of recommended malting quality varieties since 2004 were of moderate severity, and all of those recommended for feed purposes were in this category. Of the 11 varieties with high skinning severity, the following six have been recommended for malting purposes since 2004: Propino, Glassel, Shuffle, Concerto, Optic and Overture.

The Gh14 varieties included the seven control varieties common to Gh13, and a more diverse range of 116 elite spring barley varieties belonging to the AGOUEB collection. A greater number of significant differences in skinning severity were found among the Gh14 varieties assessed, with a greater number of moderately skinning varieties being significantly different from those at either end of the spectrum (Table 3) ($P < 0.05$).

Relationships between ear and grain traits and skinning severity among varieties

Ear length ranged from 6.6 cm (Felicie) to 11.6 cm (Kym); grain number from 16.1 (Tavern) to 30.4 (Sabel) and grain weight from 25.1 (Golden Promise) to 67.6 (Carvilla) mg. As grain skinning severity differed among varieties, relationships between grain skinning and the measured ear traits were investigated for Gh13 and Gh14. Only variety had a significant effect on grain skinning ($P < 0.05$). Ear length, floret number, grain number and grain weight had no significant effect on grain skinning among the 216 varieties examined.

DISCUSSION

The requirement for a reliable screen to identify varietal susceptibility to grain skinning is evidenced by the tendency for newer, malting quality varieties having high grain skinning severity in the current study. The procedure used here was based on discussion with the malting and plant breeding sectors suggesting that some popular varieties were experiencing high levels of skinning during seasonal conditions characterized by extremes in atmospheric humidity or intermittent wet and dry weather during grain filling and ripening. For example, the 1997 harvest season in southern England was noted for high skinning levels among spring barley varieties, during which high rainfall caused wetting and drying cycles during the grain filling period (Froment & South 2003). To date field experiments have been limited, but field trials imitating rainfall events during the 1997 season cited above significantly induced higher skinning levels compared to un-treated control plots (Froment & South 2003). Replication of more controlled wetting and drying conditions in the field would not be suitable for a screening procedure aimed at identifying varietal susceptibility to the condition, as variation between sites and growing seasons such as uncontrolled precipitation would preclude reliable reproduction of the method. The current study reports a more controlled and reproducible misting treatment that sufficiently increases skinning severity so that genotypic variation in susceptibility to the condition can be assessed. Although the variability in skinning severity among ears was high, the increase in mean skinning severity for each variety meant that differences among varieties could be determined, similar to the findings of Legge *et al.* (2005) who reported that the higher skinning values in malted grains corresponded with genotypes contributing the highest proportion of variance in skinning.

The absence of any correlation between grain weight and skinning severity among the 216 varieties assessed in the current study further supports the findings of Rajasekaran *et al.* (2004) and Legge *et al.* (2005) who, comparing two and 16 varieties respectively, found that differences in grain plumpness and weight did not relate to skinning levels. Although differences in grain size among varieties is not correlated with skinning, it may be that within a variety, particularly small or large grain size could lead to poor contact between the husk and caryopsis, or to mechanical stresses between husk and grain tissues, resulting in different skinning levels. If this were the case, results from protocols such as those used by industry (European Brewing Convention 2004), in which skinning severity is determined by the weight of grains with husk loss above a chosen threshold, would need to be interpreted with caution.

Previous studies have examined grain skinning among small numbers of spring barley cultivars, with contradictory reports of whether there is genetic variation in susceptibility to the condition (Aidun *et al.* 1990; Legge *et al.* 2005; Olkku *et al.* 2005; Psota *et al.* 2011). The panel of varieties assessed in the current study spans more than 50 years of spring barley breeding, and encompasses varieties with a continuous range of skinning levels. The varieties examined do not cluster into resistant and susceptible groups, suggesting that regulation of the quality of husk adhesion is likely to be complex. The covered/naked phenotype is determined by expression of the *Nud* gene, which is typically deleted in naked barleys (Taketa *et al.* 2008). However a recent study has shown that *Nud* is expressed at low levels in some Tibetan naked barley cultivars (Duan *et al.* 2015), therefore differences in the quality of husk adhesion observed among these barley varieties may in fact be regulated by differential expression of *Nud* under the misting treatment.

The misting treatment significantly increased the proportion of skinned grains without having an effect on ear length, floret number, grain number or grain weight. Therefore, skinning severity must be mediated through other physiological characteristics such as changes in the structure or composition of the lipid cementing layer. Mechanisms through which misting may induce changes in the lipid cementing layer can be inferred from literature on the effect of surface wetting on other fruit cuticles. Surface wetting, or exposure of sweet cherry fruit to high relative humidity, changes the physical properties of the cuticular membrane and results in microcracking of the surface cuticles (Knoche & Peschel 2006). Similarly, isolated tomato cuticles give different stress-strain curves depending on the relative humidity at which they are measured (Matas *et al.* 2005), and water sorption lowers the temperature at which they undergo a glass transition (Matas *et al.* 2004). The mechanical strength of the cementing layer itself may therefore be compromised by the misting treatment, impairing good quality adhesion of the husk to the caryopsis.

The fact that varieties recommended for malting typically have high skinning susceptibility illustrates that the current approaches to crop improvement, which focus on achieving higher yields on-farm, are not necessarily optimal for the entire supply chain. The misting treatment in the current study could be used by barley breeders to exploit genotypic variation in susceptibility to the malting barley defect grain skinning by selecting against those varieties that are acceptable to take forward in other traits (yield, disease resistance), but that would otherwise be let down for malting quality by high levels of grain skinning. Future investigation of changes in gene expression in the response to misting treatment, and changes in the structure and composition of the cementing layer would shed light on the fundamental mechanisms governing skinning severity, and allow more targeted breeding strategies to be implemented.

We thank A. Lowe and D. Fairgrieve for their technical assistance. We also thank Dr. W.T.B Thomas of the James Hutton Institute for provision of barley seed. This research was supported by funding from the BBSRC Crop Improvement Research Club (Project BB/J019623/1) and the Scottish Government's Rural and Environment Science and Analytical Services Division.

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Table 1. *Differences in ear length, floret number per ear, grain number per ear and grain weight among the seven control varieties*

	Concerto	Glassel	Optic	Oxbridge	Prisma	Shuffle	Tankard	S.E.D.*	Pr > F (Variety)	Pr > F (Treatment)	Pr > F (Var:Treat)
Ear length (mm)	8.7	7.8	8.7	9.2	8.1	9.2	8.3	0.27	<0.001	0.853	0.275
Floret number	29.8	28.6	28.9	28.1	29.5	28.9	26.8	0.78	0.015	0.927	0.944
Grain number	26.7	24.4	24.4	23.9	25.3	24.6	23.4	0.73	0.003	0.914	0.452
Grain weight (mg)	62	57	58	56	58	66	53	2.2	<0.001	0.509	0.810

* S.E.D. = standard error of the difference

Table 2. *Mean proportion of skinned grains among Gh13 varieties; those varieties that do not share a group are significantly different from each other*

Varieties	Mean proportion	Groups	Severity
Cork	0.000	*a*	Very low
Blenheim	0.003	*b*	Low
Adonis	0.007	*b* to *c*	Low
Appaloosa	0.007	*b* to *d*	Low
Golden Promise	0.008	*b* to *e*	Low
Annabell	0.014	*b* to *f*	Low
Aramir	0.015	*b* to *g*	Low
Astoria, Athos	0.016	*b* to *h*	Low
Brahms, Sebastian, Alexis	0.017 to 0.020	*b* to *i*	Moderate
Cocktail	0.022	*b* to *j*	Moderate
Brazil, Doyen, Heron, County	0.024 to 0.027	*b* to *k*	Moderate
Troon, Hart, Power	0.029 to 0.033	*b* to *l*	Moderate
Chad	0.033	*b* to *m*	Moderate
Beryllium	0.035	*b* to *n*	Moderate
Tyne, Kym, Garner, Drum, Century, Cooper, Cristalia	0.039 to 0.042	*b* to *o*	Moderate
Livet, Chime, Decanter, Class, Prisma, Barke, Odessa, Dallas, Vortex, Calico, Quench, Akcent, Publican, Sanette, Saloon, Fairytale, Waggon, Summit, Static, Spire	0.048 to 0.088	*b* to *p*	Moderate

Rangoon, Sabel, Atem, Chariot, Linden, Maresi, Kelim, Yard, Chronicle, Snakebite, Vivendi, Wicket, Odyssey, Prestige, Westminster, Chalice, Oxbridge, SY Taberna, Derkado, Shakira, Marthe, Riviera, Scarlett, Prague, NFC Tipple, Pasadena, Natasha, Belgravia	0.092 to 0.166	*c* to *p*	Moderate
Tavern, Camargue	0.172	*d* to *p*	Moderate
Cropton, Delibes	0.181 to 0.186	*e* to *p*	Moderate
Carafe, Triumph, Pewter, Moonshine, Aspen, Krona	0.211 to 0.298	*f* to *p*	Moderate
Panther	0.314	*g* to *p*	Moderate
Cellar	0.316	*h* to *p*	Moderate
Madras, Tankard	0.324 to 0.338	*i* to *p*	High
Propino, Glassel	0.381 to 0.389	*j* to *p*	High
Shuffle	0.399	*k* to *p*	High
Concerto, Ceylon	0.449 to 0.454	*l* to *p*	High
Braemar	0.496	*m* to *p*	High
Optic	0.509	*n* to *p*	High
Overture	0.525	*o* to *p*	High
Goldie	0.574	*p*	High

Table 3. *Mean proportion of skinned grains among Gh14 varieties; those varieties that do not share a group are significantly different from each other*

Varieties	Mean proportions	Groups	Severity
Felicie, Hassan, Zephyr	0.008 to 0.010	*a*	Low
Primera	0.013	*a* to *b*	Low
Henni	0.018	*a* to *c*	Low
Rainbow	0.020	*a* to *d*	Low
Georgie	0.021	*a* to *e*	Low
Prisma	0.025	*a* to *f*	Low
Sultan	0.042	*b* to *g*	Low
Charm	0.046	*b* to *h*	Low
Digger	0.047	*b* to *i*	Low-Moderate
Optic	0.055	*c* to *j*	Low-Moderate
Lofa Abed, Feltwell	0.055 to 0.058	*c* to *k*	Low-Moderate
Vada, Dray	0.063	*c* to *l*	Low-Moderate
Pongo	0.064	*c* to *m*	Low-Moderate
Indola	0.072	*d* to *n*	Low-Moderate
Novello	0.077	*d* to *o*	Low-Moderate
Carvilla	0.081	*e* to *p*	Low-Moderate
Fractal	0.084	*f* to *p*	Low-Moderate
Onyx	0.085	*f* to *q*	Low-Moderate
SW Scania, SW Stella	0.089	*f* to *r*	Low-Moderate
Host	0.094	*f* to *s*	Moderate
Polygena	0.095	*f* to *t*	Moderate

Brewster	0.097	*g* to *u*	Moderate
Isabella	0.105	*g* to *v*	Moderate
Acapella, Hydra	0.110	*g* to *w*	Moderate
Hopper, Cribbage	0.112	*g* to *x*	Moderate
Anais	0.113	*g* to *y*	Moderate
Lithium	0.114	*g* to *z*	Moderate
Celebra	0.120	*g* to *aa*	Moderate
Splash, Rebecca, Chieftain, Meltan, Trinidad	0.124 to 0.128	*g* to *ab*	Moderate
Alliot, Macaw, Paramount, Centurion, Mikado, Dandy, Campala, Ragtime, Torup	0.134 to 0.151	*g* to *ac*	Moderate
Acrobat, Thistle, Imber, Gundel, Turnberry, Canasta, Widre, Chaser, Concerto	0.154 to 0.163	*h* to *ad*	Moderate
Cecilia, SW Macsena	0.164	*h* to *ae*	Moderate
Dew, Akita	0.167	*i* to *ae*	Moderate
Fontana, Toucan, Crusader, Putney	0.184 to 0.192	*j* to *af*	Moderate
Proctor	0.194	*k* to *af*	Moderate
Avec, Foxtrot, Athena, Maud, Maris Mink	0.205 to 0.212	*l* to *ag*	Moderate
Velvet, Spiral, Scandium	0.214 to 0.215	*l* to *ah*	Moderate
Wren	0.219	*m* to *ah*	Moderate
Azure	0.224	*n* to *ai*	Moderate

Henley, Propino, Brise	0.228 to 0.230	*n* to *aj*	Moderate
Potter, Anaconda, Harriot, Reggae	0.235 to 0.241	*n* to *ak*	Moderate
Agenda, Rakaia, Oxbridge, Toddy	0.246 to 0.253	*o* to *ak*	Moderate
Alabama	0.264	*p* to *ak*	Moderate
Shuffle	0.275	*q* to *ak*	Moderate
Beatrix, Landlord, Maypole, Global, Glassel, Corniche	0.280 to 0.288	*r* to *ak*	Moderate
Extract, Golf	0.294 to 0.298	*s* to *ak*	Moderate
Timori	0.300	*t* to *ak*	Moderate-High
Klaxon	0.308	*u* to *ak*	Moderate-High
Ardila, Colston	0.320 to 0.325	*v* to *al*	Moderate-High
Laird	0.335	*w* to *al*	Moderate-High
Monika, Tankard	0.341 to 0.342	*x* to *al*	Moderate-High
Spike	0.345	*y* to *al*	Moderate-High
Kirsty	0.346	*z* to *al*	Moderate-High
Quartet, Silicon	0.350 to 0.361	*aa* to *al*	Moderate-High
Horizon	0.365	*ab* to *al*	Moderate-High
Berwick, Kassima	0.381 to 0.387	*ac* to *al*	Moderate-High
Rummy,Tartan	0.428	*ad* to *al*	Moderate-High
Poker	0.448	*ae* to *al*	Moderate-High
Token	0.464	*af* to *al*	High
Tabora, Heather, Skagen	0.500 to 0.514	*ag* to *al*	High
Granta	0.528	*ah* to *al*	High
Melitta	0.539	*ai* to *al*	High

Midas	0.547	*aj* to *al*	High
Macarena	0.555	*ak* to *al*	High
Clarity	0.653	*al*	High